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Research article

Effect of vaccination with a novel GnRH-based immunocontraceptive on immune responses and fertility in rats

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ABSTRACT

1. As human-wildlife conflicts increase worldwide, novel methods are required for mitigating these conflicts. Fertility control, based on immunocontraceptives, has emerged as an alternative option to lethal methods for managing wildlife.

2. Immunocontraceptives are vaccines that generate an immune response to key components of an animal's reproductive system. Some of these vaccines target the gonadotropin-releasing hormone (GnRH) and have been used successfully as contraceptives for many wildlife species. However, the need to capture animals for treatment limits the field applications of injectable vaccines. The availability of orally delivered immunocontraceptives would increase the breadth of applications of fertility control for wildlife management.

3. This study explored a new approach to developing an oral immunocontraceptive, exploiting the bioadhesive and immunologically active properties of killed *Mycobacterium avium* cell wall fragments (MAF). The MAF was conjugated to a GnRH recombinant protein called IMX294, used as a GnRH-specific immunogen.

4. An initial trial using the MAF-IMX294 conjugate provided the first evidence that an orally delivered immunocontraceptive vaccine could generate anti-GnRH antibody titres in laboratory rats.

5. Increasing the dose and frequency of vaccine administered to rats, in a second trial, enhanced the immune response, eliciting titres that reduced the proportion of females giving birth. This provided the first evidence of the contraceptive effect of an oral anti-GnRH vaccine.

6. Future work is required to further increase the immunogenic effect of the oral vaccine and to establish a dosing schedule that is effective for practical field applications.

1. Introduction

Human-wildlife conflicts, often due to overabundant wildlife populations, are increasing worldwide and have traditionally been managed through culling [1, 2, 3]. Growing antipathy for lethal methods, driven by concerns about animal welfare, human safety, and environmental impact constrains options for reducing these conflicts [4, 5, 6, 7].

Fertility control is often advocated as an alternative to lethal methods of wildlife management [8, 9, 10, 11]. Fertility control will generally achieve population reductions over a longer timescale than culling, as infertile animals are not removed [11, 12]. However, fertility control has potential advantages over lethal control. For instance, infertile animals in the population may contribute to density-dependent feedback, slowing population recovery [13]. Contraception can be particularly effective in maintaining lower population numbers after initial reduction by culling [14, 15, 16, 17]. In addition, fertility control may decrease the transmission of diseases by reducing both the number of new-born susceptible individuals [18, 19] and animal-to-animal contact during mating [20], and by minimising social perturbation compared to culling [21].

In the last 20 years, 'single-shot' injectable immunocontraceptive vaccines have been widely tested for use in wildlife management [10, 22]. These vaccines work by eliciting an immune response to proteins or hormones essential for reproduction such as the gonadotrophin releasing hormone (GnRH). GnRH is responsible for controlling reproduction in males and females by stimulating the production of the hormones that lead to ovulation and spermatogenesis. Suppressing GnRH through the

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generation of anti-GnRH antibodies prevents animals from reproducing [23, 24].

One such injectable GnRH-based immunocontraceptive vaccine, GonaCon (USDA, Pocatello, ID, USA), has proven effective in many species [3, 11, 22, 25]. Injectable immunocontraceptives rely on adjuvants containing bacterial components to enhance the longevity of the immune response to a vaccine [22, 26]. For instance, AdjuVac is an adjuvant based on killed *Mycobacterium avium*, a ubiquitous, non-pathogenic bacterium commonly found in most animal species. As most individuals have previously encountered this bacterium, using *M. avium* within a vaccine is thought to elicit a boosted response by harnessing an animal's natural exposure levels [27]. Perry et al. [28] demonstrated that the presence of *M. avium* in AdjuVac (National Wildlife Research Center, NWRC, United States), was essential for the successful contraception of black-tailed deer treated with GonaCon.

However, the utility of GonaCon and other injectable immunocontraceptives is limited by the need to capture animals for injection. The availability of oral immunocontraceptives would increase the scope of fertility control applications in wildlife. The development of oral vaccines is challenging, compared to parenteral delivery, as demonstrated by the fact that only a few orally administered vaccines currently exist [29, 30]. Rapid degradation of compounds in the digestive tract and poor permeation capacity across the intestinal mucosa constrain the effectiveness of oral vaccination [30, 31, 32].

In efforts to enhance immunogenicity against GnRH, and explore smaller compounds with increased potential for mucosal uptake, recombinant molecules containing GnRH have been developed. Osivax (formerly Imaxio) has formulated a GnRH recombinant construct called IMX294, comprising a heptameric protein (50,000 MW) containing seven copies of GnRH. This unique GnRH immunogen was effective as an injectable contraceptive in male pigs [33].

In addition to acting as an adjuvant, *M. avium* is acid resistant and known to imbed in the ileal region of the small intestine, associated with the immunologically active area of the Peyer's patch [34]. Thus, incorporating antigens into constructs such as *M. avium* may enhance mucosal uptake whilst bypassing the acid environment of the stomach.

Whilst AdjuVac contains whole killed *M. avium,* the current study pursued a novel method based on formulating *M. avium* cell wall fragments (MAF) conjugated to a putative GnRH immunogen (MAF-IMX294) as a potential GnRH vaccine for mammals [35].

Specific objectives of the studies were:

1. To assess the immunological and contraceptive effects in laboratory rats of MAF-IMX294 formulations delivered via intramuscular, oral, and nasopharyngeal routes.
2. To establish the effects of dose concentration and frequency of oral formulations of MAF-IMX294 on the immune responses and fertility of laboratory rats.

2. Methods

The laboratory rat was used as a model mammalian species for this study. Nulliparous outbred Wistar strain female rats were sourced from a registered breeder and weighed between 180 and 200g on arrival. Rats were housed in wire mesh standard breeding cages, 2–3 animals per cage, in temperature and humidity-controlled rooms on a 12 h light:12 h dark cycle and provided with *ad libitum* water and IPS 5002 pellet diet (Labdiet-IPS Ltd, London, UK). Animals were given two weeks of acclimatization before being randomly assigned to experimental groups (Table 1).

Two trials were carried out sequentially and only female rats were treated with the putative contraceptives. During each trial, blood was collected from the tail vessel of each rat prior to treatment, and again 45 days after first dosing (maximum volume 0.5 ml, using a 23G needle). For blood sampling, animals were held in restraint tubes or anaesthetised using sevoflurane. Depending on experimental group, treated animals were dosed as follows: while conscious, via oral lavage (OL) using a round-tipped metal catheter; under anaesthesia, via nasopharyngeal lavage (NP) using a pipette, or via intra-muscular injection (IM) into the back thigh using a 21G needle. Each rat received 1, 3, or 6 doses (Table 1, see details below). Three weeks after completion of the last dose, adult Wistar strain males of proven fertility were introduced into the females' cages. After a further ten days, males were removed and females were housed singly. Subsequently produced pups were removed and counted.

The effectiveness of the treatments undertaken during these trials was measured by:

1. Quantification of serum anti-GnRH antibody titres;
2. Reproductive output, expressed as number of rats giving birth and litter size.

Table 1. Experimental design used in Trial 1 and Trial 2 to test different formulations, concentrations, frequency of dosing and delivery routes of a novel immunocontraceptive vaccine (MAF-IMX294) on the fertility of laboratory rats. MAF-U = *M. avium* fragments (ultrasound), MAF-M = *M. avium* fragments (microfluidized).

Trial	Group	N	Formulation	Route	Dose	Dose frequency
1	1	10	MAF-U-IMX294	IM injection	200 μg	3
1	2	10	MAF-U-IMX294	Nasopharyngeal	50 μg	3
1	3	10	MAF-U-IMX294	Oral Lavage	500 μg	3
1	4	10	MAF-M-IMX294	IM injection	200 μg	3
1	5	10	MAF-M-IMX294	Nasopharyngeal	50 μg	3
1	6	10	MAF-M-IMX294	Oral Lavage	500 μg	3
1	7	5	MAF-M	IM injection	200 μg	3
1	8	5	MAF-M	Nasopharyngeal	50 μg	3
1	9	5	MAF-M	Oral Lavage	500 μg	3
1	10	10	GonaCon	IM injection	200 μg	1
2	1	10	MAF-IMX294 Low concentration – Low frequency	Oral Lavage	500 μg	3
2	2	10	MAF-IMX294 High concentration – Low frequency	Oral Lavage	2500 μg	3
2	3	10	MAF-IMX294 High concentration – High frequency	Oral Lavage	2500 μg	6
2	4	10	MAF-IMX294 Low concentration – High frequency	Oral Lavage	500 μg	6
2	5	10	MAF-IMX294	IM injection	200 μg	3
2	6	10	Control (not treated)	-	-	-

2.1. Trial 1. assessing the immunological and contraceptive effects of two different MAF-IMX294 formulations delivered via oral and nasopharyngeal routes in comparison to intramuscular

Fragmentation of *M. avium* whole cells was accomplished using an ultrasound method (MAF-U), or using a microfluidizer (MAF-M). For microfluidisation approximately 8–10 ml of a whole cell suspension of *Mycobacterium avium* (0.87 g/ml 0.85% saline) was transferred into 110 ml of phosphate buffered saline, pH 7.2. While stirring the solution, ≈0.5 mg of ribonuclease A (bovine pancreas, Sigma Chemicals) and 10 μl of deoxyribonuclease (bovine, Sigma) were added, followed by 1–2 ml of absolute ethanol. Using a M110L microfluidizer (Microfluidics, Westwood, MA), the solution was microfluidized by passing it three times through a G110Z reaction chamber in an ice bath at 144790 kPa with a 10 min pause between passes. The resulting total cell lysate was then centrifuged at ≈30,000 g for 30 min at 4 °C and resulting *M. avium* fragment (MAF) pellet collected, weighed, and stored frozen at -20 °C. Subsequent analysis of MAF using a particle size analyzer (Stabino, Microtrak) yielded a bimodal particle size distribution, with the first peak ranging from 0.23μm ± .075 μm (all values are mean ± SD) to 0.75μm ± 0.32 μm, max = 0.421μm ± 0.15 μm, and the second peak ranging from 1.2μm ± 0.51 μm–4.01μm ± 2.5 μm, max = 2.11μm ± 0.74 μm. The fragments of *M. avium* produced by each method were coupled to IMX294, to form the MAF-IMX294 conjugate in phosphate-buffered saline (PBS) solution. IMX294 is a tubular recombinant protein comprised of seven identical monomers, with each monomer containing a hybrid complement inhibitor chicken C4 protein fused with a GnRH peptide [36]. Following expression, the seven monomers join via cysteine-to-cysteine linkages to form a tubular heptamer (≈53 kDa).

The conjugation was achieved using a two-step EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide): N-hydroxysuccinimide ester coupling method [37]. The conjugation method used links free carboxyl groups to the primary of amine of lysines. Since both IMX 294 and MAF contain free lysines, the lysine side chains of IMX294 were capped with sulfo-N-hydroxy-acetate (SNHSA) to make the resulting conjugation unidirectional. Approximately 8.7 mg of SNHSA in 0.050 ml ultrapure water was added to a tube containing 2.84 mg ml^{-1} IMX294 in PBS. Following a 60 min incubation period, the resulting amine-capped IMX294 solution was transferred to Zeba desalting columns (7K MWCO, 5 ml, Thermo Scientific) and centrifuged at 1000 g for 2 min to remove excess SNHSA. Resulting eluate was collected, yielding ≈1 ml of stabile amine-capped IMX294.

To each 1 ml vial of capped IMX294, 0.4 mg of sulfo-NHS and 0.6 mg of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) combined in 0.10 ml PBS was added, followed by a 30 min incubation. The resulting activated IMX294 was eluted through a Zeba desalting column as previously described, and immediately added to 20 mg MAF in PBS and reacted for 2 h. The conjugation was quenched by adding 0.025 of a hydroxylamine solution, then transferred to dialysis cassete (Slide-A-Lyzer® G2, Thermo Scientific) and dialyzed overnight. The following morning, the conjugate solution was removed from the cassette and stored at 4 °C. Chemical conjugation was demonstrated by bioassay in pilot study in which a conjugate was administered to one group while an unconjugated mixture was given to a second group. Only the conjugated material produced an anti-GnRH response.

Using a spectrophotometric assay, MAF lysine content was determined to be ≈ 1% of total mass. Given a starting conjugation MAF mass of 20 mg and a lysine to IMX294 ratio of 1:1, the theoretical maximum conjugate IMX294 content would be about 1.4 μmol. All conjugates and treatment solutions were prepared at the NWRC.

Six treatment groups of rats were used to test the two formulations of conjugate (MAF-U-IMX294 and MAF-M-IMX294) compared to negative controls (MAF-M only) with treatments delivered by intramuscular injection, nasopharyngeal lavage, or oral lavage (Table 1). All groups were administered a prime dose followed by two boosters at 15 day intervals, with the exception of a positive control group that received a single injection of GonaCon.

2.2. Trial 2. establish the contraceptive effectiveness of different orally delivered doses and frequency of MAF-IMX294

The aim of Trial 2 was to optimise the formulation of the oral vaccine and thereby increase the potential to reduce rat fertility, by a) increasing the concentration of MAF-IMX294 from the 1000 μg/ml of solution used in Trial 1–5000 μg/ml; and b) increasing the frequency of administration from three to six evenly spaced doses over the same time period of 30 days. Nasopharyngeal delivery groups were not included in Trial 2 as Trial 1 indicated that similar immune responses could be generated through oral delivery, which is more practical for field use.

Microfluidized *M. avium* fragments (MAF-M) were used in Trial 2, as no differences were apparent in the anti-GnRH antibodies produced by the MAF obtained by ultrasound or by microfluidization in Trial 1. Additionally, for IM treatments the MAF-IMX294 conjugate was emulsified with a mineral oil plus surfactant solution (90% w/w Sigma M1180 USP light grade mineral oil; 10% w/w Sigma M8819 mannide monoleate surfactant), as used in GonaCon, in order to maximize the antibody response to GnRH [38]. Treatment groups receiving three doses were administered a prime dose followed by two boosters at 15 day intervals. Treatment groups receiving six doses were administered a prime dose followed by five boosters at 5 day intervals. Controls were not dosed and were used to compare the reproductive output with that of other groups.

2.3. Analyses

Anti-GnRH antibody titres in serum samples were quantified using an indirect ELISA technique outlined in Miller et al. [39], Levy et al. [40] and Bender et al. [41], adapted for the laboratory rat using rabbit anti-rat IgG, followed by goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma Chemical Co., St. Louis, MO, USA). A post-treatment serum sample was considered positive for anti-GnRH antibodies if the optical density value was greater than the mean optical density plus two standard deviations of the control (pre-treatment sample) values for each respective dilution.

Differences in the proportions of females giving birth and litter sizes were analysed using Fisher's exact, Mann-Whitney U, and Kruskal-Wallis tests in SPSS for Windows (Version 25, IBM Corp., 2013). One-tailed tests were used when the direction of the effect could be predicted, otherwise two-tailed tests were employed. Differences in anti-GnRH antibody titre levels between experimental groups were examined by ordinal logistic regression using the package "MASS" [42] in R version 3.4.3 [43] (odds ratio (OR) and their 95% confidence intervals (CI) are reported). An alpha level of 0.05 was used for all statistical tests. A receiver operating characteristic (ROC) analysis was used to quantify how accurately the anti-GnRH antibody titre level can be used to discriminate between two states, "fertile" and "infertile". A ROC curve was created based on the trade-off between sensitivity and specificity at different cut-off points (or thresholds) of a diagnostic test with a continuous outcome (our measure of anti-GnRH antibody titre level in a female) compared to a gold standard test (whether the female was later observed to give birth). A sensitivity ≥95% was used as the criterion to derive the threshold, based on anti-GnRH antibody titres, above which rats were predicted to be infertile.

The study was approved in the UK by the Animal and Plant Health Agency's Animal Welfare Ethical Review Body and carried out in accordance with the U.K. Animals (Scientific Procedures) Act 1986.

3. Results

In Trial 1, one animal in Group 4 died at the beginning of the trial (suspected liver pathology), and another in Group 5 died for unknown causes. In Trial 2, one animal in Group 6 died for unknown causes.

3.1. Anti-GnRH antibody titres in relation to treatment

3.1.1. Trial 1

MAF-IMX294 generated anti-GnRH antibody titres detectable 45 days after first dosing via all three delivery routes (intramuscular injection, nasopharyngeal lavage, and oral lavage, Table 2). These titres ranged from 1k (i.e. maximum dilution at which antibodies were detected being 1:1,000) to 1024k. The method of MAF fragmentation did not affect the immunogenicity of the vaccine as no significant differences were observed in the odds of having higher anti-GnRH antibody titres between MAF-U and MAF-M groups (OR: 0.97 (95% CI: 0.38–2.43), $p = 0.94$). Consequently, data were pooled within delivery route for the analysis of breeding success presented below: Groups 1 and 4- MAF-IMX294 intramuscular injection (IM); Groups 2 and 5- MAF-IMX294 nasopharyngeal lavage (NP); Groups 3 and 6- MAF-IMX294 oral lavage (OL). Data from the control groups (MAF-M only IM, NP, and OL) were also pooled as no females in these three groups exhibited detectable anti-GnRH antibody titres.

The GonaCon positive control treatment (Group 10), generated the highest antibody titres (512k-2048k, Table 2). In the MAF-IMX294 treatments, 95% (18 out of 19) of rats injected with MAF-IMX294 (Group 1 and 4) had antibody titres, ranging between 4k and 1024k. Of rats administered MAF-IMX294 via the oral (Group 3 and 6) or nasopharyngeal (Group 2 and 5) route 70% (14 out of 20) and 53% (10 out of 19) respectively displayed titres, all ≤128k.

Titres generated by the IM formulations were significantly higher than those generated through oral treatment (OR: 26.1 (95% CI: 6.7–118.2), $p < 0.001$). There was no significant difference in titre levels of females between nasopharyngeal delivery and oral delivery groups (OR: 0.98 (95%CI: 0.3–3.1), $p = 0.98$).

3.1.2. Trial 2

In Trial 2, the oral dosing of MAF-IMX294 successfully elicited antibody titres in 17 of 40 treated females, ranging between 32k and 512k (Table 2). Increasing the dose frequency and concentration enhanced the treatment effect as significantly higher titres were observed at high concentration formulations (OR = 11.6 (95% CI: 2.7–60.9), $p = 0.002$) and higher dose frequencies (OR = 5.2 (95% CI: 1.3–24.5), $p = 0.02$). No anti-GnRH antibody titres were observed in the ten females from the oral MAF-IMX294 low concentration, low frequency group (Table 2, Trial 2 - Group 1). This was in contrast to the results from an equivalent treatment dose in Trial 1, where 14 out of 20 females (70%) displayed titres (Trial 1 - Groups 3 and 6, Table 2).

The highest titres in Trial 2 were observed in the IM MAF-IMX294 treatment group (Group 5), where all females (n = 10) displayed tires ≥1024k. The inclusion of an additional mineral oil and surfactant emulsion in the IM MAF-IMX294 formulation in Trial 2 (Group 5, Table 2) increased the immune response compared to the IM MAF-IMX294 without additional emulsion tested in Trial 1 (Groups 1 and 4, Table 2).

3.2. Litter production in relation to treatment

3.2.1. Trial 1

In Trial 1, no difference in number of females producing litters (Kruskal-Wallis, H = 2, N = 15, 2 d.f., P = .368) or litter size (Kruskal-Wallis, H(2) = 2.81, $p = 0.25$) was found between the three MAF only control groups, hence these groups were pooled for further analyses. No females from the GonaCon group (Group 10) produced litters.

The number of females that produced a litter in any of the three MAF-IMX294 groups did not differ significantly from that of the pooled control group (Table 3, Fisher's exact two-sided; all $p \geq 0.2$). For all females that produced litters in the MAF-IMX294 groups, litter size did not differ from that of the control group (Kruskal-Wallis, H (3) = 1.81, $p = 0.61$). These results indicate that, regardless of delivery route, treatment with MAF-IMX294 did not appear to affect fertility in this trial.

3.2.2. Trial 2

In Trial 2, the proportion of rats that produced litters in groups administered oral MAF-IMX294 (Groups 1–4) did not differ from that of the controls (Group 6) (Fisher's exact one-sided, $p = 0.43$, Table 3). However, six out of ten females in the high concentration, high frequency group (Group 3) did not produce litters. The proportion of rats producing litters in Group 3 (4/10) was significantly lower than that of the other three oral treatment groups in Trial 2 combined, where 7/10, 9/10 and 7/10 females produced litters in Groups 1, 2 and 4 respectively (Fisher's exact one-sided, $p = 0.04$). This was also lower than the number of females that produced litters in the negative control group (Group 6) in

Table 2. The number of female rats with anti-GnRH antibody titres (presented as the highest 1:X,000 dilution at which antibodies were detected 45 days after first dose was administered in Trial 1 and Trial 2. OL = Oral lavage; NP = Nasopharyngeal; IM = Intramuscular injection; LC = Low concentration; HC = High concentration; NT = No titre detectable.

Trial	Group	Treatment	N	Titre (1:X,000)													% with titre
				NT	1	2	4	8	16	32	64	128	256	512	1024	2048	
1	1	IM MAF-U-IMX294	10	1						1	4	1	1	2			90
1	4	IM MAF-M-IMX294	9	0			1			1	2	2	2		1		100
1	2	NP MAF-U-IMX294	10	5					2		1	2					50
1	5	NP MAF-M-IMX294	9	4		1		3		1							55.56
1	3	OL MAF-U-IMX294	10	4	1	1		3		1							60
1	6	OL MAF-M-IMX294	10	2		1	2	2	2		1						80
1	10	IM GonaCon	10	0										2	2	6	100
1	7	IM MAF-M only	5	5													0
1	8	NP MAF-M only	5	5													0
1	9	OL MAF-M only	5	5													0
2	1	OL MAF-IMX294 LC x 3	10	10													0
2	2	OL MAF-IMX294 HC x 3	10	4							1	4		1			60
2	3	OL MAF-IMX294 HC x 6	10	4							1		1	4			60
2	4	OL MAF-IMX294 LC x 6	10	5						3	1			1			50
2	5	IM MAF-IMX294 x 3	10	0											1	9	100
2	6	Control	9	9													0

a) "MAF-U" and "MAF-M" groups were pooled as no differences were found in anti-GnRH antibody titres between these groups.
b) Negative control groups (MAF only - IM, OL, and NP) were merged as no differences were found in anti-GnRH antibody titres between these groups.

Table 3. Number of female rats breeding in each group and mean litter size (plus standard deviation, SD) of females that bred in Trial 1 and Trial 2. Abbreviations as in Table 2.

Trial	Group	Treatment	Doses	n	n bred	% bred	Mean (SD) litter size of breeding rats
1	1	IM MAF-U-IMX294	3	10	7	70	8.43 (2.77)
1	4	IM MAF-M-IMX294	3	9	7	77.8	12.71 (2.31)
1	2	NP MAF-U-IMX294	3	10	9	90	10.77 (2.66)
1	5	NP MAF-M-IMX294	3	9	7	77.8	10.29 (2.81)
1	3	OL MAF-U-IMX294	3	10	7[a]	70	11.71 (3.49)
1	6	OL MAF-M-IMX294	3	10	9	90	10.22 (4.52)
1	10	IM GonaCon	1	10	0	0	-
1	7	IM MAF-M only	3	5	5	100	9.4 (2.94)
1	8	NP MAF-M only	3	5	4	80	7 (3.24)
1	9	OL MAF-M only	3	5	5	100	10.6 (4.32)
2	1	OL MAF-IMX294 LC	3	10	7	70	9.86 (2.85)
2	2	OL MAF-IMX294 HC	3	10	9	90	9.67 (2.18)
2	3	OL MAF-IMX294 HC	6	10	4	40	9.33 (3.06)[b]
2	4	OL MAF-IMX294 LC	6	10	7	70	9.14 (3.76)
2	5	IM MAF-IMX294	3	10	0	0	0.00 (0.00)
2	6	Control	-	9	7	78	9.29 (3.64)

[a] Unknown if 1 female bred.
[b] n = 3, litter size unknown for 1 female.

which two out of nine rats did not litter, although this difference was not statistically significant (Fisher's exact one-sided p = 0.12). The lack of significance may have been due to small sample size. When the proportion of rats that produced litters in Group 3 (4/10) was compared to that of the combined negative control group of Trial 1 (14/15), with a relatively larger sample size, the difference between groups was significant (Fisher's exact one-sided p = .007).

There was no difference in litter sizes between females that produced litters in Group 3 (high concentration, high frequency) and rats in the negative control group (Group 6) (Table 3, Mann-Whitney U = 58, p = 0.136).

3.3. Breeding in relation to anti-GnRH antibody titre

The ROC curve indicates that titres of ≥256k are highly associated with infertility across all trials (Figure 1).

Using the threshold of 256k, the levels of producing litters in relation to titre were examined for all rats dosed with oral and injectable formulations of MAF-IMX294 in Trial 1 and Trial 2. In 108 MAF-IMX294 treated rats, 69 animals exhibited anti-GnRH antibody titres. Anti-GnRH antibody titres greater than 1024k indicated complete infertility, as none of the 15 rats with titres of 2048k produced litters. Titres between 256k and 1024k were associated with a reduction in the proportion of females producing litters. Of females with no titres, 76.9% produced litters (30 out of 39), compared to 84.8% of females breeding with titres between 1k and 128k (39 out of 46), and 17.3% of females breeding with titres ≥256k (four out of 23). The difference in number of individuals producing litters with a titre of ≥256k compared to females with no titre was significant (Fisher's exact one-sided, p < 0.001). Across trials, there was no evidence that anti-GnRH antibody titres lower than 256k impaired fertility as there was no significant difference found in the proportion of MAF-IMX294 treated females producing litters between those with no titre and those with titres between 1k and 128k (Fisher's exact one-sided, p = 0.19).

Across orally delivered treatments of MAF-IMX294 in Trial 2, seven of the females given oral doses of MAF-IMX294 generated titres of 256k or more and ten females exhibited titres between 32k and 128k. None of the females with titres ≥256k produced litters, which was a significantly lower proportion than that of females with titres below this threshold, in

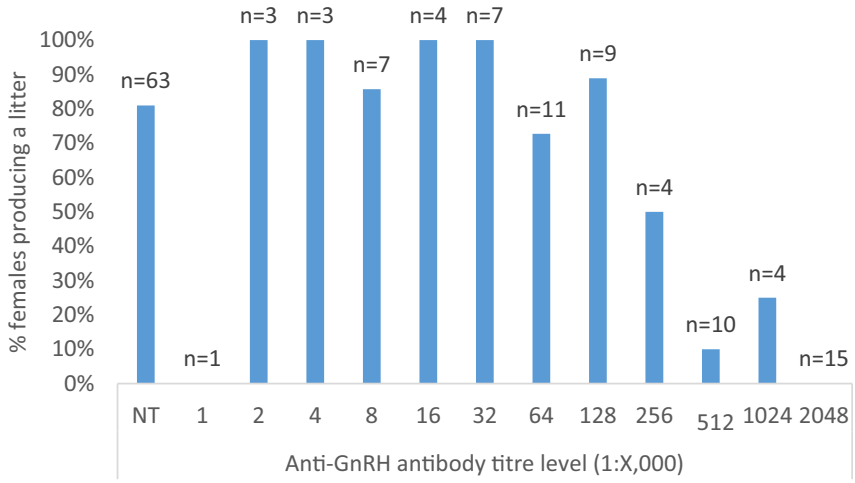

Figure 1. Percentage of female rats that produced litters in relation to anti-GnRH antibody titre (1: X,000) across all treatment groups in Trial 1 and Trial 2. NT = No detectable titres. n = sample size.

Table 4. Mean litter size of female rats that produced litters (bred) in relation to anti-GnRH antibody titre across all treatment groups in Trial 1 and Trial 2.

Titre (1:X,000)	N bred	Mean litter size	SD
0	51	9.72[(a)]	3.31
1–128	39	10.46	3.7
>128	4	9.5	1.29
Total	94	10.02	3.42

[(a)] n = 50, litter size unknown for one female.

which nine out of ten produced litters (Fisher's exact one-sided, $p < 0.001$).

For all treatments, litter size did not differ significantly between females with no titre, those with titres of 1-128k, that did not appear to influence the probability producing litters, and those with titres of ≥256k that reduced this probability (Table 4, Kruskal-Wallis, $H(2) = 1.67$, $p = 0.43$).

4. Discussion

This study provided the first evidence of anti-GnRH antibody titres generated by oral delivery of an immunocontraceptive vaccine (Trial 1). This represents a major breakthrough in eliciting an immune response to a small 'self' hormone, like GnRH, through an oral route. Despite the immune response, the titres induced from oral treatments in Trial 1 were insufficient to affect female fertility. However, Trial 2 indicated that increasing both the dose concentration and the frequency of oral administration improved the immune response. This trial provided the first evidence of reduced fertility arising from oral dosing with an anti-GnRH immunocontraceptive vaccine. In addition, the results of both trials suggested that anti-GnRH antibody titres of ≥256k are associated with infertility in female rats.

In Trial 2, the lack of difference in litter size between the females in Group 3 that did produce litters and the control group was likely due to the treated females not responding to the vaccine. All four breeding females had no detectable anti-GnRH titres, indicating that their reproduction was entirely unaffected by treatment.

The absence of antibody titres in rats in the low concentration-low frequency group (Group 1) in Trial 2 was unexpected, as an equivalent formulation elicited immune responses in 14 out of 20 rats (Group 3 and 6) in Trial 1. This discrepancy may have been due to variation in batches of reagents or plates and clearly limited the comparisons that could be drawn between trials but not within trials. Although direct comparisons between species need to be interpreted with care, the strength of the anti-GnRH immune response required to affect fertility in the rat demonstrated in this study (256k or above) is higher than observed in several other species such as white-tailed deer and feral horses, for which titres of 64k typically impair fertility following treatment with GonaCon [27, 44].

Mathematical models have suggested that in wild rodents a minimum of 60–70% of both sexes need to be rendered infertile for at least three generations for fertility control to have an effect at population level [45, 46]. As the most effective oral dosing formulation in this study indicated infertility in 60% of treated rats, the next steps will focus on exploring methods for increasing the immunogenicity of the oral vaccine. Further investigation should consider methods of vaccine protection through the gastrointestinal tract. In parallel, using bioadhesive compounds could potentially maximise vaccine uptake by providing an intimate contact between these drugs and the mucosa, thus maintaining a high concentration at the absorptive surface for an extended period [47]. Future studies should test dosing schedules that are effective over a timescale commensurate with a practical baiting strategy as well as species-specific methods to deliver contraceptives in baits.

Overall, this study demonstrated that MAF-IMX294 induced immune responses sufficient to reduce fertility in rats through both injectable and oral routes. This suggests that the MAF-IMX294 has potential for the formulation of an oral anti-GnRH based immunocontraceptive vaccine for mammals. As human-wildlife conflicts increase, oral immunocontraceptives would represent a significant tool to control overabundant populations of wildlife. For some key target species, such as the wild boar (*Sus scrofa*), systems are already available for the delivery of baits containing contraceptives [48]. However, before oral contraceptives can be added to the toolbox of methods to manage wildlife, new studies will be required to test doses and frequency of dosing for each species, to optimise bait formulation, and to evaluate the feasibility and costs of using this method for population control.

Declarations

Author contribution statement

Massei G: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Cowan D: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Eckery D: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Mauldin R, Rochaix P, Hill F: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Gomm M: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Pinkham R: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Miller L. A: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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